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Cholesterol Uptake by *Propionibacterium freudenreichii*

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Abstract. Strains of *Propionibacterium freudenreichii* were tested for growth and cholesterol uptake in brain–heart infusion (BHI) medium supplemented with pleuropneumonia-like organism (PPLO) serum fraction as the cholesterol source. Stirred cultures of *P. freudenreichii* reduced the medium's cholesterol content by 50% or more after 10–14 days of incubation at 32°C. Cholesterol uptake by the propionibacteria did not require strictly anaerobic conditions or the presence of bile acids. Up to 70% of the cholesterol removed from the medium could be recovered by solvent extraction from washed cells of *P. freudenreichii*.

The discovery of epidemiological relatedness between serum cholesterol levels and the incidence of coronary heart disease [11] implied that potential benefits may be associated with reduced dietary cholesterol intake. This has led to an increased interest in the development of low-cholesterol dairy foods and the evaluation of microorganisms of dairy food fermentations for metabolic activities involving cholesterol. In addition, there is interest in the microbial transformation of cholesterol present in dairy foods to coprostanol [14] or other derivatives that may have limited absorbability in the intestinal tract [1].

Ingestion of certain dairy foods fermented with selected cultures of streptococci or lactobacilli may lead to the reduction of serum cholesterol levels in humans [8, 12] and animals [7, 13, 15–17, 19]. Although the general mode of action involved in this phenomenon has not been elucidated, in some cases direct microbial action on cholesterol and related compounds was clearly established [5, 6].

Propionibacterium freudenreichii is a Gram-positive, nonsporulating, nonmotile, aerotolerant chemoorganotroph with primary habitats in dairy products [3]. Its importance in food fermentations is owing to its essential role in the ripening of Swiss-style cheeses. During the fermentation process, *P. freudenreichii* is primarily responsible for the conversion of lactate produced by companion species of lactic acid bacteria (Streptococcus sp., Lactobacillus sp.) to propionate, acetate, and CO_2. Another important function is the production of proline, mostly as the result of peptidase and, to a limited extent, proteolytic and biosynthetic activities [9, 10]. The cumulative effect of these metabolic functions results in the development of physical and flavor properties characteristic of the finished cheese. Other important uses of *P. freudenreichii* include the production of vitamin B_{12} [20] and the control of psychrotropic spoilage bacteria in various foods [4].

Here we describe the uptake of cholesterol from laboratory media by *P. freudenreichii*, a well-known microorganism in specific dairy fermentations that has not been reported to have capacity for interaction with cholesterol.

Materials and Methods

Bacterial strains. *Propionibacterium freudenreichii* 6207, 8262, 9614, 9615 (American Type Culture Collection, Rockville, MD), 3524 and 4327 (Northern Regional Research Center, U.S. Dept. of Agriculture, Peoria, IL) were maintained in brain–heart infusion (BHI) broth medium (Difco Laboratories[1], Detroit, Michigan) by weekly subculture and incubation for 24 h at 32°C before storage at 4°C. The purity of the cultures was routinely monitored by microscopic examination.

Cholesterol uptake media. BHI broth or tryptic soy glucose broth (TSB, Difco) media were prepared at 2× strength, dispensed in 25-ml Corex tubes with screwcaps (10 ml per tube), and sterilized by autoclaving at 121°C for 15 min. After cooling, a defined amount of pleuropneumonia-like organism (PPLO) se-

[1] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Table 1. Uptake of cholesterol by different strains of *Propionibacterium freudenreichii*

Strain	Cholesterol removed from medium (%)
ATCC 6207	70
ATCC 8262	64
ATCC 9614	52
ATCC 9615	58
NRRL 3524	52
NRRL 4327	60

Initial cholesterol concentration was 325 μg/ml. Cultures were grown in BHI broth supplemented with PPLO serum. Incubation was at 32°C for 12 days.

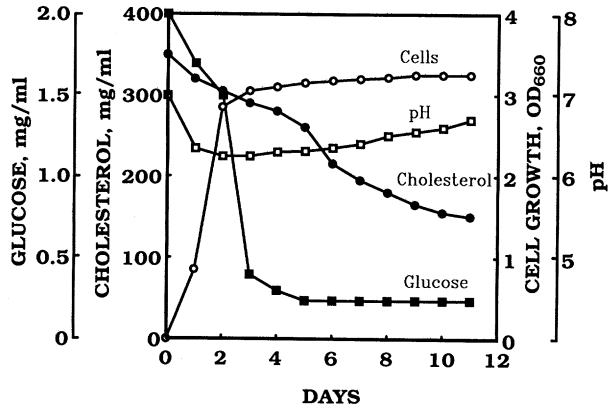

Fig. 1. Uptake of cholesterol by *Propionibacterium freudenreichii* from BHI-PPLO medium. Symbols: 0 cells (OD_{660}); 0 cholesterol; ■ glucose; □ pH.

rum fraction (Difco) was added as the source of cholesterol to each tube under aseptic conditions. Inoculation was with 0.2 ml of 24-h bacterial growth, and the final volume of each tube was adjusted to 20 ml with sterile H_2O to yield 1× strength for both BHI and TSB medium formulations. Tubes were fitted with magnetic stirring bars and placed on a Quad Drive System platform (Belco Biotechnology, Vineland, New Jersey). Incubation was at 32°C and 90 rpm stirrer platform setting.

Measurement of cholesterol depletion. Cells growing in BHI-PPLO or TSB-PPLO medium were removed by centrifugation for 5 min at 16,000 *g* in an Eppendorf microcentrifuge (Brinkman Instruments, Westbury, New York). The cholesterol content of supernatants was determined in 25- to 100-μl samples by an enzymatic colorimetric method (Cholesterol CII Kit, Wako Pure Chemical Industries Ltd., Osaka, Japan).

Cholesterol content of cell pellets was measured by extraction with ethyl acetate, centrifugation to sediment cell debris, and evaporation of aliquots of the solvent phase to dryness. Residues were dissolved in *n*-propanol and assayed with the Cholesterol CII enzyme kit. Alternatively, cholesterol content of cell extracts was measured by the ferric chloride method [18].

Bacterial growth and bile tolerance. Bacterial growth was monitored by the OD at 660 nm (OD_{660}) in a spectrophotometer. Samples were diluted 1 : 10 with water. Bile tolerance of cultures was tested by adding oxgall (Difco) to BHI broth to 0.5% final concentration.

Glucose assays. During cholesterol depletion trials, glucose fermentation was followed by measuring the amount of glucose of cell-free broth with glucose (HK) reagent (Sigma Diagnostics, St. Louis, Missouri) according to the manufacturer's instructions.

Thin layer chromatography (TLC). Ethyl acetate extracts of cells, cell-free growth media, and PPLO serum samples were evaporated to dryness. Residues were redissolved in ethyl acetate, spotted on SIL G-25 silica gel plates (Brinkmann), and developed in a 95 : 5 (vol/vol) chloroform–ethyl acetate solvent system. TLC plates were sprayed with a copper sulfate–phosphoric acid reagent [2], and after charring at 130°C for 5 min in a convection oven they were photographed under UV light.

Chemicals. All biochemicals, reagents, and solvents were commercial products of the highest analytical purity.

Results and Discussion

Capacity for cholesterol uptake. All six strains of *Propionibacterium freudenreichii* showed the capacity for removing cholesterol from BHI-PPLO and TSB-PPLO media. The amount of cholesterol removed during the 12-day incubation period varied from 50% to 70% (Table 1). The addition of bile acids (oxgall) to the medium had no effect on culture growth. Furthermore, the presence of bile acids was not a prerequisite for cholesterol uptake by propionibacteria. The maintenance of strictly anaerobic conditions was also unnecessary for cholesterol uptake to take place. These findings were in sharp contrast with results obtained by others with selected strains of lactobacilli that require the presence of bile acids and strictly anaerobic conditions for cholesterol assimilation [6]. In the case of *P. freudenreichii*, interaction with cholesterol is apparently regulated by an entirely different mechanism.

Dynamics of cholesterol uptake. The pattern of growth, cholesterol uptake, glucose dissimilation, and pH changes was established for each test organism. A representative summary of these results is given for *P. freudenreichii* 6207 in Fig. 1. Culture growth (OD_{660}) was rapid and reached near maximum after 72 h under experimental conditions, although continued slight increases in cell density were observed throughout the entire test period. Dissimilation of glucose also proceeded rapidly,

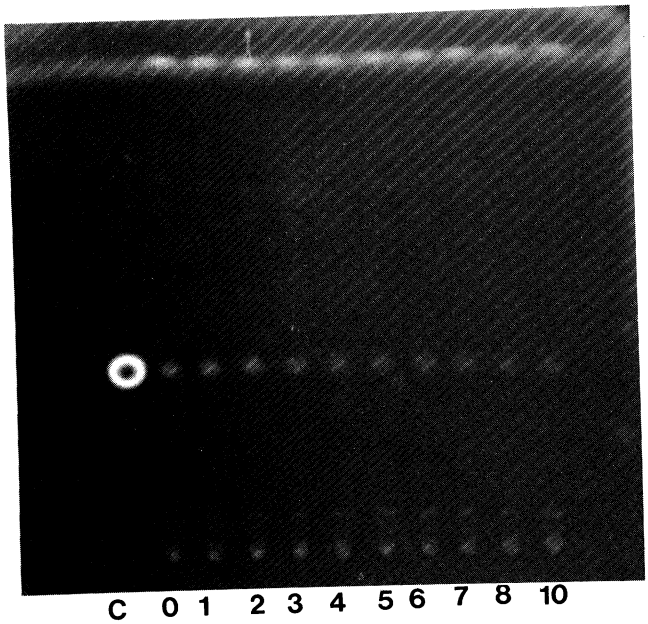

Fig. 2. TLC analysis of cholesterol uptake from BHI-PPLO medium. Cell-free samples (300 μl) were extracted with ethyl acetate. Residue in solvent phase was analyzed on SIL G-25 silica gel plates in chloroform–ethyl acetate (95 : 5), vol/vol). Steroid-like compounds were visualized with a $CuSO_4$-H_3PO_4 reagent, under UV light. C, cholesterol standard (in days).

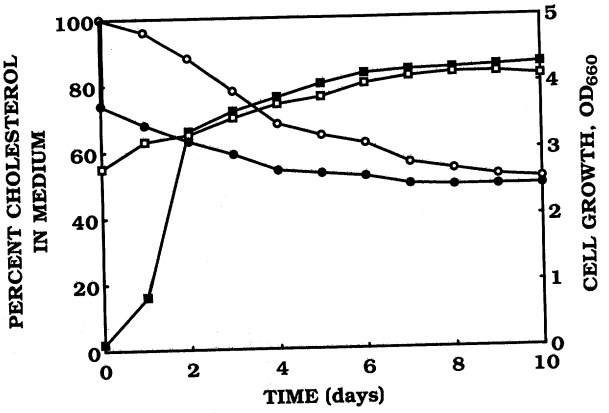

Fig. 3. Influence of cell density (OD_{660}) on cholesterol uptake. Culture age at the time of adding PPLO serum to BHI medium was 0 h (○) or 72 h (●); ■ and □ indicate corresponding growth curves at OD_{660}.

Table 2. Effect of cholesterol (PPLO) concentration on cholesterol uptake by *Propionibacterium freudenreichii* ATCC 6207

Initial cholesterol concentration (μg/ml)	Percent cholesterol removed at day		
	3	6	10
84	55	71	74
162	35	44	52
224	25	40	48
292	23	37	51
422	23.5	37.5	52

Conditions: BHI medium (1× final strength); 32°C.

with more than 90% depleted from the medium within 48 h from the time of inoculation.

The enzymatic analysis of cell-free supernatants indicated a gradual disappearance of cholesterol from the medium. Maximum cholesterol loss was as high as 70% after 10–12 days of incubation. However, the average cholesterol loss was between 55% and 60% in repeated trials. Analysis of ethyl acetate extracts of cell-free broth samples by TLC also showed the gradual loss of extractable cholesterol (Fig. 2). Experiments with other strains of *P. freudenreichii* yielded essentially similar information on the dynamics of cholesterol depletion from PPLO serum.

Effect of PPLO concentration. The effect of cholesterol concentratoin on uptake by *P. freudenreichii* was evaluated by varying the amount of sterile PPLO serum added to the test media. The rate of cholesterol depletion appeared to be inversely proportional to the medium's cholesterol level during the first 5–6 days of the incubation period (Table 2). However, with the exception of the lowest initial cholesterol concentration (84 μg/ml) used, the amount of cholesterol removed by the end of 10 days was approximately 50% of the total cholesterol present, independent of the initial concentration. These results implied that *P. freudenreichii* interacts preferentially with certain classes of cholesterol lipoprotein complexes in PPLO serum, the identity of which is not known at the present.

Effect of microbial population density. The dynamics of cholesterol uptake by *P. freudenreichii* was further characterized by varying the culture's population density (OD_{660}) at the time of PPLO addition. The results of these experiments are summarized in Fig. 3. With the increase of initial cell density, the proportion of PPLO serum cholesterol immediately depleted (within 10 min of PPLO addition) from the medium also increased, with only more moderate losses of cholesterol to follow during the rest of the incubation period. This strongly suggested that the uptake of cholesterol by *P. freudenreichii*, at least in part, may be explained in

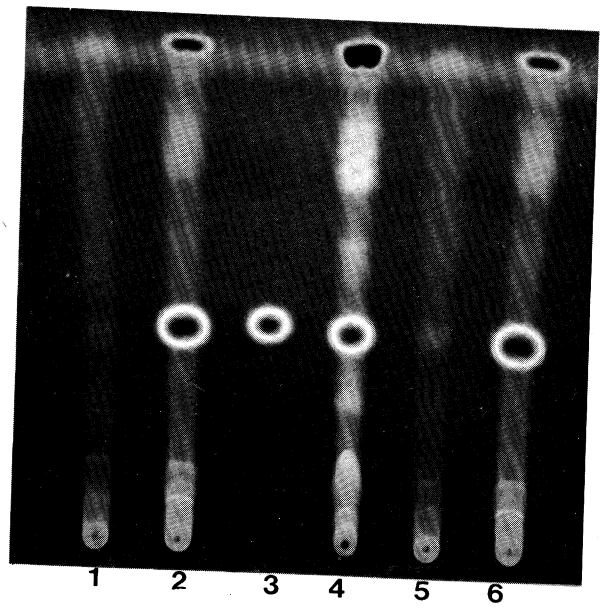

Fig. 4. TLC analysis of *P. freudenreichii* cell extracts. Ethyl acetate extracts of cell pellets of strain 6207 were analyzed on SIL G-25 plates in a $CHCl_3$–ethyl acetate, 95 : 5 (vol/vol) solvent system. 1, extract of strain 6207 grown in BHI; 2, extract 6207 cells grown in BHI-PPLO; 3, cholesterol standard; 4, ethyl acetate extract of PPLO serum; 5, extract of strain 8262 grown in BHI; 6, extract of 8262 cells grown in BHI-PPLO.

terms of a binding phenomenon, possibly involving extracellular polysaccharides (slime) that some propionibacteria are known to produce [10] or specific membrane-bound receptor proteins capable of interaction with certain cholesterol lipoprotein complexes present in PPLO serum. After the initial drop, further incremental losses in cholesterol content may be owing to the continued slow increases in cell density during the test period, as already mentioned.

Fate of cholesterol depleted by propionibacteria. Ethyl acetate extracts of *P. freudenreichii* cells grown in the absence or presence of PPLO serum cholesterol were compared by TLC analysis (Fig. 4). It was apparent that many PPLO serum components reacting positively with the copper sulfate–phosphoric acid reagent could be recognized in the solvent extracts of cells.

The enzymatic determination of cell-bound cholesterol in *P. freudenreichii* established that approximately 80% of the PPLO serum cholesterol lost from the medium could be recovered from cell pellets. This finding indicated that the extent of actual cholesterol dissimilation by propionibacteria was at best limited.

In conclusion, different strains of *P. freudenreichii* were found to interact with PPLO serum cholesterol, leading to a gradual depletion of cholesterol from laboratory media. Since much of the cholesterol may be recovered, apparently unaltered, from cell pellets by solvent extraction, cholesterol uptake may be the result of interaction between cell envelope components or cell envelope-bound biopolymers of the propionibacteria and the cholesterol lipoprotein complexes of PPLO serum. The characterization of the mechanism of this interaction is currently in progress.

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